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PRINCIPAL INVESTIGATOR: Eileen M. Rogan, Ph.D.

CONTRACTING ORGANIZATION: Garvan Institute of Medical Research
Darlinghurst NSW 2010 Australia

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Darlinghurst NSW 2010 Australia

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This project focuses on the role of c-Myc in mediating estrogen actions in breast epithelial cells, specifically to determine the contribution of c-Myc to downstream molecular and cellular events after estrogen stimulation. The rationale for this investigation is, i) the established intrinsic role of estrogens in mammary gland development and etiology of breast cancer and the proven therapeutic efficacy of antiestrogens in breast cancer, and ii) compelling evidence that has implicated the cell cycle regulatory molecules c-Myc and cyclin D1 in estrogen-induced mitogenesis. Our experimental approach involves ectopic expression of wild-type, mutant and dominant-negative variants of c-Myc to modulate c-Myc function and assay the necessity for various functional domains of c-Myc in mediating estrogen stimulation of breast cancer cell proliferation. To date, good progress has been made. An excellent model has facilitated observation of molecular events downstream from estrogen and/or c-Myc in breast cancer cells: induction of various cell cycle regulatory proteins, formation and activation of regulatory protein kinase complexes, phosphorylation of crucial target substrates and S phase progression have been documented and characterized, resulting in a high quality publication. Future work includes completion of these initial studies, extension of *in vitro* studies to identify functional domains of c-Myc required for its estrogen-mediating effects and ultimately, *in vivo* studies in reconstituted murine mammary epithelial glands.

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Eileen Logan 24/07/99

PI - Signature Date

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INTRODUCTION

This project focuses on the role of c-Myc in estrogenic actions in breast epithelial cells, specifically with the aim of determining the contribution of c-Myc to various downstream molecular and cellular events after estrogen stimulation. The rationale for this investigation lies in i) the established intrinsic role of estrogens in mammary gland development and etiology of breast cancer and the proven therapeutic efficacy of antiestrogens in breast cancer, and ii) compelling evidence that has implicated the cell cycle regulatory molecules c-Myc and cyclin D1 in estrogen-induced mitogenesis. Our experimental approach involves ectopic expression of wild-type, mutant and dominant-negative variants of c-Myc to modulate c-Myc function and assay the necessity for various functional domains of c-Myc in mediating estrogen stimulation of breast cancer cell proliferation. To date, good progress has been made on experimental tasks set for the initial 12 months of this project. An excellent model previously established in this laboratory has facilitated observation of various molecular events downstream from estrogen and/or c-Myc in breast cancer cells: induction of various cell cycle regulatory proteins, formation and activation of regulatory protein kinase complexes, phosphorylation of crucial target substrates and S phase progression have been documented and characterized, resulting in a high quality publication on which the principal investigator is second author. Future work includes completion of these initial studies and extension of *in vitro* studies to identify functional domains of c-Myc required for its estrogen-mediating effects and ultimately, *in vivo* studies in reconstituted murine mammary epithelial glands.

Abbreviations

Cdk	cyclin-dependent kinase
p21	Cdk inhibitor $p21^{Waf1/Cip1}$
p27	Cdk inhibitor $p27^{Kip1}$

BODY – ANNUAL SUMMARY

- **The hypotheses addressed in the initial stages of this project were as follows:**

1. Induction of c-Myc expression can precipitate a cascade of molecular events which results in cell cycle progression in breast cancer cells *in vitro* mimicking the effects of estrogen treatment.

2. Multiple pathways for estrogen action exist and may converge at the point of activation of cyclin E-Cdk2. We predict that c-Myc activation of the cyclin E-Cdk2 complex will proceed via the same mechanism as induced by estrogen.

- **Progress**

    - **Work Task 1 (as per *Statement of Work*)**
    - **c-Myc and estrogen reversal of anti-estrogen arrested breast cancer cells.**

To address these hypotheses, we have used clonal mammary carcinoma cell lines which express either cyclin D1 or c-Myc under a zinc-inducible promoter (pΔMT) (previously-derived in this laboratory by transfection of these constructs into a clonal line of steroid-responsive MCF-7 cells [Prall *et al.*, 1997]. Inducibility of these proteins in this system was confirmed. When these cells are pretreated with a pure steroidal estrogen antagonist (ICI 182780 10 nM) for 48 hours, S phase (DNA synthesis) reaches a minimum. Subsequent treatment with 50 μm zinc induces cyclin D1 or c-Myc expression to similar levels as those observed after estrogen (Estradiol [E2] 100 nM) "rescue". In contrast, cyclin D1 and c-Myc levels do not increase following zinc-treatment of vector-alone transfected control cells. Further investigations revealed that inducible expression of either gene was sufficient to induce progression of the cells into S phase: this finding implied that the induction of one gene can override antiestrogen-mediated suppression of the other. Our preliminary data suggested some divergence between the molecular pathways induced by cyclin D1 and c-Myc and the molecular basis for this became an ongoing focus of our attention. For example, only cyclin D1 induction was accompanied by generation of active cyclin D1-

Cdk4 complexes and the induction of expression of either cyclin D1 or c-Myc alone did not lead to significantly increased expression of the other protein.

We therefore performed further "rescue" experiments in this model and carefully documented downstream molecular events such as, induction of other cell cycle regulatory proteins (eg. cyclins E and D1, Cdk inhibitors p21 and p27), formation and activation of regulatory protein kinase complexes (Cdk4/cyclin D1 and Cdk2/cyclin E), phosphorylation of crucial target substrates (pocket proteins – pRB, p107) and S phase progression. The precise composition of *active* versus *inactive* p21/cyclin E-Cdk2 complexes was a particular focus of our attention after we demonstrated that following either estrogen or c-Myc treatment of anti-estrogen arrested cells, a minor fraction of cellular cyclin E-Cdk2 complexes underwent a shift from low to high molecular weight accompanied by acquisition of specific kinase activity, which preceded pRB phosphorylation and S phase progression.

Gel filtration chromatography was utilised extensively for these analyses: this technique facilitates fractionation of proteins on the basis of molecular weight, allowing observations of shifts in protein complex size and analysis of both composition and biological activity of protein complexes over a range of molecular weights. Cell lysates from antiestrogen-arrested, "estrogen-" or "c-Myc-rescued" cells were subjected to gel filtration chromatography followed by immunoprecipitation with monoclonal antibodies directed against proteins of interest. In addition to these analyses of protein complex composition, specific *in vitro* kinase activity of p21-, cyclin E- and Cdk2-associated complexes was also assayed over a range of filtration fractions. A combination of this methodology followed by dissociation of precipitated p21/cyclin E-Cdk2-associated complexes, separation by gel electrophoresis and Western blotting for candidate molecules was used to identify proteins associated with the increase size and activity of cyclin E-Cdk2 kinase complexes preceding estrogen and c-Myc-stimulated S phase progression.

Essentially we found that inducible expression of either c-Myc or cyclin D1 was sufficient for S-phase entry in cells previously arrested in G1 phase by pretreatment with a potent estrogen antagonist. c-Myc expression was not accompanied by increased cyclin D1 expression or Cdk4 activation, nor was cyclin D1 induction accompanied by increases in c-Myc. Expression of c-Myc or cyclin D1 was sufficient to activate cyclin E-Cdk2 by promoting the formation of high-molecular-weight complexes lacking the cyclin-dependent kinase inhibitor p21, as has been described following estrogen treatment. Most interestingly, this was accompanied by an association between active cyclin E-Cdk2 complexes and hyperphosphorylated p130, identifying a previously undefined role for p130 in estrogen action. These data provide evidence for distinct c-Myc and cyclin D1 pathways in estrogen-induced mitogenesis which converge on or prior to the formation of active cyclin E-Cdk2-p130 complexes and loss of inactive cyclin E-Cdk2-p21 complexes, indicating a physiologically relevant role for the cyclin E binding motifs shared by p130 and p21.

Our methods and findings are comprehensively documented in the publication arising from this work – Prall *et al.* (1998) *c-Myc or cyclin D1 mimic estrogen effects on cyclin E-Cdk2 activation and cell cycle re-entry. Mol. Cell. Biol., 18: 4499-4508* **(Appendix 1).** Note that this was a combined study in which the roles of both cyclin D1 and c-Myc in estrogen-induced breast cancer mitogenesis were studied in parallel. The c-Myc part of the study was performed by the principal investigator.

Ongoing work

Since beginning this study, further details about the transcriptional regulation activities of c-Myc have been elucidated. c-Myc is a bHLH/LZ (basic helix-loop-helix/leucine zipper) transcriptional regulator which forms heterodimeric complexes with another bHLH/LZ protein, Max. Myc/Max complexes are now known to both activate and repress transcription via the amino terminus of c-Myc which contains two highly conserved Myc box regions: MbI (Myc box I, amino acids 45-63) is necessary for transcriptional *activation;* and MbII (amino acids 129-141) for transcriptional *repression,* c-Myc-induced *cell proliferation* and *transformation.* Several genes involved in growth have now been identified as possible targets of c-Myc including *p53, ornithine decarboxylase* (*ODC*), *cad* and *cdc25A* which are transcriptionally activated, and the growth arrest genes *gas1, C/EBP* and *gadd45* which are transcriptionally repressed.

Since MbII is necessary for c-Myc-induced cell proliferation we predict that c-Myc repressed genes are likely to be involved in both estrogen- and Myc-induced cyclin E-Cdk2 activation and G_1-S phase progression. In an extension of our original research plan and with collaboration with others in this

laboratory, we plan to test this by examining the ability of various c-Myc mutants to promote cell cycle progression in antiestrogen-arrested MCF-7 cells. To this end we have now obtained the following c-Myc mutants: 1) cMycDMbII, which has a deleted MbII domain; 2) MycS, a naturally occuring translational form of c-Myc, which lacks 100 amino-terminal amino acids, including the MbI domain; and 3) full-length c-Myc. Plasmids containing these cDNAs will be transiently transfected into antiestrogen-arrested MCF-7 cells with a marker plasmid (eg. green fluorescent protein (GFP), and rescue into S-phase will be analyzed by dual parameter flow cytometry. Alternatively, cDNAs will be cloned into the pΔMT plasmid (the zinc-inducible metallothionein promoter used in the previous experiments), stably transfected MCF-7 clones selected and proteins expressed by addition of zinc to culture media. This approach will enable us to identify the domains of c-Myc that are *sufficient* for Myc-induced G_1-S phase progression in antiestrogen-arrested breast cancer cells. This should facilitate identification of relevant downstream targets of c-Myc by eliminating possible confounding genes that are regulated by full-length c-Myc but that are not essential for cell cycle progression. In parallel experiments aimed at identifying candidate c-Myc targets associated with estrogen-induced cell cycle progression, we will investigate the expression patterns of potential Myc target genes by Northern and Western analysis following estrogen stimulation or ectopic c-Myc expression in MCF-7 cells. Such genes include *p53, ODC, cad, cyclin D1, cyclin E, p21, p27, cdc25A, gas1, C/EBP* and *gadd45* (we already have the cDNAs to these genes).

- Work Task 2 (as per *Statement of Work*)
- Study of the necessity for c-Myc in estrogen rescue from anti-estrogen arrest in breast cancer cells using inducible dominant-negative c-Myc mutants.

The original proposal described use of the "Tet-Off" inducible gene expression system for these experiments. However, preliminary experiments that were conducted prior to cloning of dominant negative c-Myc into this expression system, revealed an array of technical and logistic problems with this system for our purposes. Consequently, we have experimented with an alternative novel protein expression system "pTAT" (expression of proteins of interest in bacterial cells followed by protein harvest and addition to mammalian cells of interest in culture [Nagahara *et al.*, 1998). This expression system has also posed some technical challenges - eg. difficulty in reproducing adequate protein entry into cultured breast epithelial cells. However, we have interacted with the original authors of this method regarding technical problem-solving and are confident of resolving such problems. To date, our progress in this area includes construction of pTAT plasmids encoding the relevant dominant negative and wild-type c-Myc plasmids and expression of those proteins in bacteria. We have also developed a Fugene 6 transfection protocol capable of transfecting plasmids into ~25% of antiestrogen-arrested MCF-7 cells, and this technique may also be used in combination with dual parameter flow cytometry for these experiments.

- Work Task 3 (as per *Statement of Work*)
- **Study of *in vivo* requirement for c_Myc expression in mammary gland development.**

The *in vivo* experimental work has not yet been commenced and will follow completion of at least preliminary experiments described in Task 2 of this proposal.

References

Prall O.W.J., Sarcevic, B., Musgrove E.A., Watts, C.K.W., and Sutherland, R.L. (1997) Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclinE-Cdk2. *J. Biol. Chem.*, 272:10882-10894.

Nagahara H., Vocero-Akbani A.M., Snyder E.L., Ho A., Latham D.G., Lissy N.A., Becker-Hapak M., Ezhevsky S.A., and Dowdy S.F.(1998) Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. *Nature Med.*, 4(12):1449-52.

ACCOMPLISHMENTS/REPORTABLE OUTCOMES

Key Research Outcomes:

- Experimental evidence of sufficiency of either c-Myc or cyclin D1 for S-phase entry in cells previously arrested in G1 phase by pretreatment with a potent estrogen antagonist (i.e. ability of these molecules to mimic estrogen in this system)

- Identification of distinct pathways for c-Myc and cyclin D1 in mediation of estrogen actions in breast epithelial cells, converging on activation of cyclin E-Cdk2 kinase complexes via a novel mechaism. This mechanism involves formation of active cyclin E-Cdk2-p130 complexes and loss of inactive cyclin E-Cdk2-p21 complexes, indicating a physiologically relevant role for the cyclin E binding motifs shared by p130 and p21.

Publications:

Completed research described in this project report has contributed to the following peer-reviewed original articles or reviews:

1. Prall O.W.J., Rogan E.M., Musgrove E.A., Watts, C.K.W., and Sutherland, R.L. (1998) c-Myc or cyclin D1 mimic estrogen effects on cyclin E-Cdk2 activation and cell cycle re-entry. *Mol. Cell. Biol.*, 18: 4499-4508.

2. Prall, O.W.J., Rogan , E.M., and Sutherland, R.L. (1998) Estrogen regulation of cell cycle progression in breast cancer cells. Invited review, *J. Steroid Biochem. Mol. Biol.*, 65:169-174.

3. Watts, C.K.W., Prall O.W.J., Carroll J. S., Wilcken N. R. C., Rogan E. M., Musgrove E. A., and Sutherland R. L.. (1999) Antiestrogens and the cell cycle. Invited review, In: Jordan V. C. and Furr, B. J. (Eds.), Antiestrogens and antiandrogens. Humanan Press, In Press.

Technical Achievements:

- We have procured plasmids expressing c-Myc variants of interest for ongoing studies of requirements for functional domains of c-Myc in mediating estrogen actions.
  For example:
  - cMycDMbII, which has a deleted MbII domain
  - MycS, a naturally occuring form of c-Myc lacking 100 N-terminal aas, including the MbI domain

- We have constructed pTAT plasmids encoding the relevant dominant negative and wild-type c-Myc plasmids and expression of those proteins in bacteria.

- We have developed a Fugene 6 transfection protocol capable of transfecting plasmids into ~25% of antiestrogen-arrested MCF-7 cells. This technique may be used in combination with dual parameter flow cytometry for studies using c-Myc mutant constructs.

APPENDIX 1 - STATEMENT OF WORK

TASKS

1. **c-Myc and estrogen reversal of antiestrogen-arrested breast cancer cells. (Months 1-18)**

   Experiments in MCF-7 clonal cell line expressing zinc-inducible c-Myc (off plasmid vector p_MT-c-Myc) to characterize the ability of c-Myc to mimic estrogen actions. Documentation of downstream molecular and cellular events - eg. by Western blotting, Northern blotting, gel filtration chromatography, immunoprecipitations, *in vivo* radioactive labeling of proteins, *in vitro* kinase assays, assessment of cell cycle parameters by flow cytometry. Comparison with vector-alone (pΔMT) transfected controls.

   Analysis of the mechanism of activation of cyclin E/cdk2 complexes following estrogen or c-Myc rescue from antiestrogen arrest. Dissection of cyclin E/cdk2/p21-associated complexes by gel filtration chromatography/immunoprecipitation/western blotting/silver staining/*in vivo* labeling of proteins/*in vitro* kinase assays, kinase complex activation assays, etc.

2. **Study of c-Myc necessity for estrogen rescue from antiestrogen-arrest in breast cancer cells using inducible dominant negative c-Myc. (Months 6-24)**

   Construction of vectors for expression in "Tet-Off" inducible expression system in MCF-7 cells. (Cloning of c-*zip* cDNA into pTRE plasmid vector for expression of c-Myc dominant negative protein.)

   Transfection of TtA-expressing MCF-7 cells (Clontech) with pTRE-*c-zip* and selection of transfected clones.
   Confirmation of expression of dominant negative mutant by PCR and Western blotting.
   Confirmation of inhibition of c-Myc function by transactivation assays.

   Experiments in inducible dominant negative model of c-Myc inhibition to assess requirement for c-Myc expression in estrogen-induced proliferation. Comparison with estrogen-treated vector-alone (pTRE) transfected controls.

   Documentation of downstream molecular and cellular events - eg. by Western blotting, Northern blotting, gel filtration chromatography, immunoprecipitations, *in vivo* radioactive labeling of proteins, *in vitro* kinase assays, assessment of cell cycle parameters by flow cytometry.

3. **Study of *in vivo* requirement for c-Myc expression in mammary gland development. (Months 18-36)**

   Establishment of techniques for murine mammary tissue reconstitution experiments. eg. Optimize retroviral transfection efficiency in primary cells.

   Construction of INA retroviral expression vectors (INA-c-*zip*) for c-Myc dominant negative expression in primary mammary epithelial cells.

   Structural and histological analysis of mammary gland development and differentiation in transgenic mammary tissue. Gross and microscopic examination of transgenic mammary tissue. β -galactosidase staining to localize transgenic glandular tissue.

   Analysis of c-Myc dysfunction on mammary gland differentiation during puberty, pregnancy and lactation.

MOLECULAR AND CELLULAR BIOLOGY, Aug. 1998, p. 4499–4508 Vol. 18, No. 8
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c-Myc or Cyclin D1 Mimics Estrogen Effects on Cyclin E-Cdk2 Activation and Cell Cycle Reentry

OWEN W. J. PRALL, EILEEN M. ROGAN, ELIZABETH A. MUSGROVE, COLIN K. W. WATTS, AND ROBERT L. SUTHERLAND*

Cancer Research Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, New South Wales 2010, Australia

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Estrogen-induced progression through G_1 phase of the cell cycle is preceded by increased expression of the G_1-phase regulatory proteins c-Myc and cyclin D1. To investigate the potential contribution of these proteins to estrogen action, we derived clonal MCF-7 breast cancer cell lines in which c-Myc or cyclin D1 was expressed under the control of the metal-inducible metallothionein promoter. Inducible expression of either c-Myc or cyclin D1 was sufficient for S-phase entry in cells previously arrested in G_1 phase by pretreatment with ICI 182780, a potent estrogen antagonist. c-Myc expression was not accompanied by increased cyclin D1 expression or Cdk4 activation, nor was cyclin D1 induction accompanied by increases in c-Myc. Expression of c-Myc or cyclin D1 was sufficient to activate cyclin E-Cdk2 by promoting the formation of high-molecular-weight complexes lacking the cyclin-dependent kinase inhibitor p21, as has been described, following estrogen treatment. Interestingly, this was accompanied by an association between active cyclin E-Cdk2 complexes and hyperphosphorylated p130, identifying a previously undefined role for p130 in estrogen action. These data provide evidence for distinct c-Myc and cyclin D1 pathways in estrogen-induced mitogenesis which converge on or prior to the formation of active cyclin E-Cdk2-p130 complexes and loss of inactive cyclin E-Cdk2-p21 complexes, indicating a physiologically relevant role for the cyclin E binding motifs shared by p130 and p21.

Estrogenic steroids elicit mitogenic responses in a variety of cell types, particularly those of female reproductive tissues, including uterus and mammary gland tissues. In addition, estrogens have well-described mitogenic actions on neoplastic breast epithelial cells both in vivo (55) and in vitro (25), and this effect has been linked to the established role of estrogens in the development and progression of the majority of human breast cancers (16). Estrogenic steroids, e.g., 17β-estradiol (E_2), stimulate resting (G_0-phase) cells to enter the cell cycle and accelerate G_1-S-phase progression (23, 58). Advances in the understanding of molecular mechanisms controlling cell cycle progression (31, 50, 51, 66) have identified cyclin-dependent kinases (CDKs) as potential targets of E_2-induced mitogenesis (2, 12, 39, 42).

Sensitivity to mitogenic stimulation is limited to G_1 phase of the cell cycle, transit through which is regulated by the activities of Cdk4, Cdk6, and Cdk2. These CDKs are activated by cyclin binding: Cdk4 and Cdk6 by D-type cyclins (50) and Cdk2 by cyclin E (22). Additional control of cyclin-CDK activity is achieved by phosphorylation/dephosphorylation of specific residues conserved among CDKs and by interaction with two families of CDK inhibitors: the INK4 family, of which $p16^{INK4A}$ is prototypic, and the $p21^{WAF1,CIP1,SDI1}$/$p27^{KIP1}$/$p57^{KIP2}$ family (reviewed in references 31 and 51). Other factors, such as the activity of Cdc25 phosphatases that catalyze the removal of inhibitory phosphates on CDKs (31), identify a further degree of complexity in CDK regulation. G_1-phase progression induced by a variety of mitogens is associated with specific effects on these CDK regulatory mechanisms (38, 49). Current evidence suggests that G_1-phase cyclin-CDK complexes promote S-phase entry by phosphorylating key protein substrates that include pRB (the product of the retinoblastoma susceptibility gene) and other members of the pocket protein family, p107 and p130. Hypophosphorylated pocket proteins bind and repress the transcriptional activity of the E2F/DP family of proteins, and phosphorylation of these pocket proteins by CDKs releases E2F/DP, with consequent activation of transcription of genes whose products are required for S-phase progression (46, 66).

E_2-induced G_1-S-phase progression in MCF-7 breast cancer cells has recently been linked to increased cyclin D1 expression, cyclin D1-Cdk4 complex formation, and cyclin D1-Cdk4 activation (2, 12, 39, 42), suggesting that cyclin D1 may mediate E_2 effects. This is supported by studies demonstrating that overexpression of cyclin D1 in breast cancer cells is sufficient to overcome antiestrogen-induced G_1-phase arrest (67) and also by the prevention of E_2-induced G_1-S-phase progression following microinjection of cyclin D1 antibodies or the Cdk4 inhibitor $p16^{INK4A}$ (protein or cDNA) (26). However, mice carrying a null mutation of both cyclin D1 alleles exhibit normal mammary gland ductal development and pregnancy-related uterine hyperplasia (11, 53). These processes are E_2 dependent, indicating the presence of cyclin D1-independent mechanisms by which E_2 can stimulate cell proliferation.

Another target of estrogen action on cell proliferation is the proto-oncogene product c-Myc, which is rapidly induced in target cells following E_2 treatment (10, 32). c-Myc antisense oligonucleotides inhibit E_2-stimulated breast cancer cell proliferation (64), and therefore c-Myc is likely to play a key role in estrogen action. In fibroblasts, c-Myc is both necessary and sufficient for G_1-S-phase progression (17). In these cells, activation of conditional alleles of c-*myc* is followed by the activation of both cyclin D1-Cdk4 and cyclin E-Cdk2 (36, 48, 56). A number of mechanisms have been defined for cyclin E-Cdk2 activation by c-Myc and include conversion of cyclin E-Cdk2 complexes to forms that can be activated by Cdc25 phosphatase (56), an increase in cyclin E protein levels (20, 36), and

* Corresponding author. Mailing address: Cancer Research Program, Garvan Institute of Medical Research, 384 Victoria St., Darlinghurst, Sydney, New South Wales 2010, Australia. Phone: 61-2-9295 8322. Fax: 61-2-9295 8321. E-mail: r.sutherland@garvan.unsw.edu.au.

prevention of the association between the CDK inhibitor p27 and cyclin E-Cdk2 (36, 63). In MCF-7 breast cancer cells, E_2 treatment also activates cyclin E-Cdk2 (12, 39, 42). We and others have presented evidence that activation of cyclin E-Cdk2 results from the failure of such complexes to bind the CDK inhibitor p21 (39, 42). Active cyclin E-Cdk2 complexes induced by E_2 in MCF-7 cells are relatively deficient in both p21 and p27 (42). Furthermore, following E_2 treatment there is a decrease in inhibitory activity toward cyclin E-Cdk2. This inhibitory activity is predominantly due to p21, not p27 (39, 42), and is accompanied by a decrease in the ability of p21 to associate with recombinant cyclin E-Cdk2 (42). While the mechanisms underlying the redistribution of p21 are undefined, there are parallels with the effect of c-Myc on p27 and cyclin E-Cdk2 association. It is therefore possible that E_2-induced activation of cyclin E-Cdk2 via p21 redistribution is mediated by the preceding increase in c-Myc expression.

To evaluate the potential contributions of c-Myc and cyclin D1 to the proliferative effect of E_2, we constructed MCF-7 cell lines that expressed either protein under the control of the Zn-inducible metallothionein promoter. Zn-induced expression of c-Myc or cyclin D1 was, like that of E_2, sufficient to promote S-phase entry in cells that had been previously arrested in G_1 phase by the antiestrogen ICI 182780. Expression of c-Myc or cyclin D1 also mimicked the effect of E_2 on activation of cyclin E-Cdk2 via formation of active cyclin E-Cdk2-p130 complexes at the expense of inactive cyclin E-Cdk2-p21 complexes.

MATERIALS AND METHODS

Antibodies. Monoclonal antibodies (in parentheses) directed against the following proteins were used: c-Myc (9E10; American Type Culture Collection, Manassas, Va.), cyclin D1 (DCS-6; Novacastra Laboratories Ltd., Newcastle-upon-Tyne, United Kingdom), cyclin E (HE12; Santa Cruz Biotechnology Inc., Santa Cruz, Calif.), pRB (G3-245; PharMingen, San Diego, Calif.), p21 (catalog no. C24420; Transduction Laboratories, Lexington, Ky.), p27 (catalog no. K25020, Transduction Laboratories), and glutathione *S*-transferase (GST) (B-14; Santa Cruz Biotechnology).

Rabbit polyclonal antibodies against cyclin E (C-19), Cdk4 (H-22), Cdk2 (M2), p21 (C-19), p107 (C-18), and p130 (C-20) and their corresponding immunogenic peptides were obtained from Santa Cruz Biotechnology. Rabbit antiserum to cyclin D1 has been described previously (34). A purified rabbit polyclonal antibody against a pRB-derived peptide (phosphorylated on the amino acid corresponding to Ser-780) was a gift from Y. Taya, National Cancer Center Research Institute, Tokyo, Japan, and has been recently described (21).

Plasmid construction. Plasmid pΔMTcycD1, which has a metal-inducible metallothionein promoter (6) upstream of the cDNA sequence of human cyclin D1, has been described previously (33). The same procedure was used to clone a cDNA encoding human c-Myc into the *SalI* site of pΔMT (pΔMTmyc). The integrity of this construct was confirmed by sequencing the entire coding region. c-Myc cDNA was obtained from Jerry Adams, Walter and Eliza Hall Institute, Parkville, Victoria, Australia.

Transfection, cell culture, and DNA flow cytometry. MCF-7 cells were obtained from the EG & G Mason Research Institute (Worcester, Mass.) and were maintained as previously described (57). MCF-7.7, a clonal MCF-7 cell line derived by limiting dilution (8), was transfected with either pΔMT, pΔMTmyc, or pΔMTcycD1 by electroporation or calcium phosphate precipitation procedures that have been previously described (33, 67). Expansion of individual G418-resistant colonies generated clonal cell lines containing either pΔMT (MCF-7.7mt), pΔMTmyc (MCF-7.7myc), or pΔMTcycD1 (MCF-7.7D1). Pools of G418-resistant cells were also generated by expanding multiple colonies together.

Exponentially proliferating cells were growth arrested by pretreatment for 48 h with 10 nM steroidal antiestrogen ICI 182780 {7α-[9-(4,4,5,5,5-pentafluropentylsulfinyl)nonyl]estra-1,3,5,(10)-triene-3,17β-diol; from Alan Wakeling, Zeneca Pharmaceuticals, Macclesfield, United Kingdom} and then treated with either 100 nM E_2 as described previously (42) or Zn (as $ZnSO_4$) as described previously (67). Unless otherwise indicated, the final concentration of Zn was 50 μM. Vehicle controls for E_2 and Zn were absolute ethanol and water, respectively. In some experiments, the specific Cdk2 inhibitor roscovitine (Calbiochem-Novabiochem, Alexandria, New South Wales, Australia) was added directly to cell culture medium 30 min prior to the addition of either E_2, Zn, or vehicle. Working dilutions of roscovitine were prepared in dimethyl sulfoxide at 1,000-fold the required final concentration in cell culture medium. Analysis of cell cycle phase distribution by DNA flow cytometry was performed as described previously (67), with minor modifications: the final concentration of ethidium bromide was 50 μg/ml, mithramycin was omitted, and RNase A was added to a final concentration of 0.4 mg/ml 1 to 24 h prior to analysis.

Immunoblotting, immunoprecipitation, and protein kinase assays. Immunoblotting and immunoprecipitation were performed as described previously (42). Kinase assays for Cdk4 and cyclin E-associated activity were performed by the methods described previously (42), with minor modifications to the Cdk4 assay as follows. Cdk4 complexes were immunoprecipitated by incubating lysates containing 400 μg of protein with 5 μl of a rabbit polyclonal Cdk4 antibody (H-22; Santa Cruz Biotechnology) for 1 h at 4°C. The complexes were recovered by the addition of 7.5 μl of protein A-Sepharose beads (Zymed, San Francisco, Calif.) per sample and further incubation for 30 min at 4°C. The final kinase reaction mixture contained 10 μg of bovine serum albumin.

Gel filtration. Cell lysates were fractionated on a HiLoad 16/60 Superdex 200 column (Pharmacia Biotech, Uppsala, Sweden) as previously described (42). Proteins were eluted at 1.2 ml/min at 4°C in a buffer consisting of 20 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA, 0.1% (vol/vol) β-mercaptoethanol, and 0.01% (vol/vol) Tween 20. The column void volume was ~45 ml, and 10 3-ml fractions were collected between 55 and 84 ml (termed fractions 1 to 10). Column calibration was performed as described previously (42).

Binding of p21 to recombinant cyclin E-Cdk2. Assays designed to determine the ability of p21 in cell lysates to bind to recombinant GST-cyclin E-Cdk2 were performed by incubating either recombinant GST-cyclin E-Cdk2 complexes (42) or GST with cell lysates for either 2 h at 4°C or 30 min at 30°C. GST-cyclin E-Cdk2 complexes were retrieved with glutathione-agarose beads added for 1 h at 4°C and washed three times with lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% [vol/vol] glycerol, 1% [vol/vol] Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM NaF, 1 mM dithiothreitol). p21 bound to recombinant GST-cyclin E-Cdk2 was detected following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis.

RESULTS

Antiestrogen-induced G_1-phase arrest can be reversed by inducible expression of either c-Myc or cyclin D1. E_2-induced G_1-S-phase progression in MCF-7 cells is associated with increased expression of c-Myc (10, 42) and cyclin D1 (2, 12, 39, 42). To assess the ability of either protein to promote G_1-S-phase progression, MCF-7.7 cell lines that contained stably integrated c-Myc cDNA (MCF-7.7myc) or cyclin D1 cDNA (MCF-7.7D1) under the control of the Zn-inducible metallothionein promoter were derived. Pooled and clonal cell lines were growth arrested by 48 h of pretreatment with the steroidal antiestrogen ICI 182780 and tested for inducible expression of c-Myc and cyclin D1 following treatment with 50 μM Zn. For both MCF-7.7myc and MCF-7.7D1 cell lines, there was a wide range of both basal and Zn-inducible expression of the exogenous proteins (Fig. 1A). Zn treatment had no detectable effect on c-Myc or cyclin D1 expression in control cell lines transfected with vector alone (MCF-7.7mt).

The ability of c-Myc or cyclin D1 to rescue cells arrested in G_1 phase by ICI 182780 was then tested in multiple cell lines. Induced expression of c-Myc or cyclin D1 in these cell lines was sufficient to promote G_1-S-phase progression (Fig. 1B). In all cell lines, there was a good correlation between ectopic protein expression and S-phase entry (Fig. 2B and data not shown). The kinetics of S phase entry were studied in the clonal cell lines MCF-7.7D1.13 (D1.13) and MCF-7.7myc.3 (myc.3) because these cell lines had the lowest basal expression of c-Myc and cyclin D1, and high expression of the ectopic genes was achieved with relatively low concentrations of Zn (Fig. 2B). Following E_2 or Zn treatment of D1.13 cells, there were substantial increases in the proportion of cells in S phase by 15 to 16 h and maximum levels were reached between 21 and 24 h (Fig. 1C). The kinetics of S-phase entry were similar in myc.3 cells, although S-phase entry was somewhat earlier, with substantial increases in the proportion of cells in S phase by 12 h and maximum levels reached at 16 to 18 h. Interestingly, the doubling time for all myc cell lines was less than that for D1

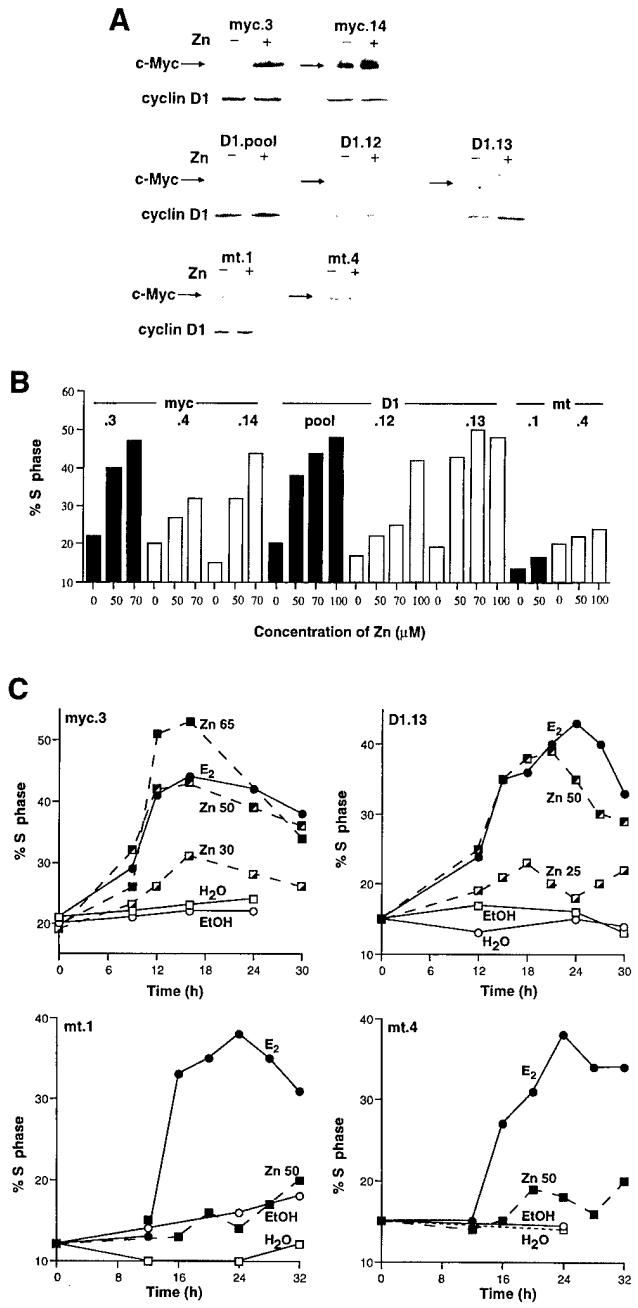

FIG. 1. Generation of MCF-7 cell lines with Zn-inducible c-Myc or cyclin D1. MCF-7.7 cell lines stably transfected with the Zn-inducible pΔMT vector containing c-Myc cDNA (myc), cyclin D1 cDNA (D1), or no cDNA (mt) were growth arrested for 48 h with 10 nM antiestrogen ICI 182780. (A) Cells were treated at time zero with either 50 μM Zn (+) or vehicle (−). After 6 to 8 h, cell lysates were prepared and immunoblotted with antibodies against c-Myc and cyclin D1. (B) Cells were treated at time zero with the indicated concentration (micromolar) of Zn. Cells were harvested (18 h for myc cells; 21 h for D1 and mt cells) and stained for DNA content, and the proportion of cells in S phase was determined by flow cytometry. (C) Cells were treated at time zero with either the indicated concentration (micromolar) of Zn, 100 nM 17β-estradiol (E_2) or vehicle (ethanol [EtOH]). At intervals thereafter, cells were harvested and stained for DNA content, and the proportion of cells in S phase was determined by flow cytometry.

and mt cell lines (data not shown), which may indicate slightly enhanced cell proliferation due to leaky c-Myc expression from the metallothionein promoter. As described above, Zn-induced S-phase entry was concentration dependent in D1.13 and myc.3 cells and in both, 50 μM Zn stimulated degrees of S-phase entry similar to that induced by E_2 (Fig. 1C). E_2 treatment of control cell lines (mt.1 and mt.4) caused an increase in the proportion of cells in S phase similar to that in the other cell lines, but 50 μM Zn treatment had little effect on G_1-S-phase progression (Fig. 1C), consistent with the negligible effect of Zn on c-Myc and cyclin D1 protein expression in these control clones (Fig. 1A). Unless stated otherwise, subsequent experiments used the clonal MCF-7.7 cell lines myc.3, D1.13, and mt.4 and rescue from ICI 182780 arrest with either 50 μM Zn or 100 nM E_2.

Increased expression of c-Myc failed to induce cyclin D1, and vice versa. c-Myc has been proposed to either increase (5), decrease (20, 37), or have no effect on (18, 19, 54) cyclin D1 gene expression in fibroblasts. E_2-induced expression of c-Myc protein by 30 to 120 min (42, 64) and cyclin D1 by 120 to 240 min (2, 12, 39, 42) in MCF-7 cells is consistent with the possibility that E_2 induction of c-Myc is a prerequisite for expression of cyclin D1. This was investigated by comparing the temporal changes in expression of these proteins during G_1-phase progression following E_2 treatment with their expression following Zn-induced expression of either c-Myc or cyclin D1. E_2 treatment increased expression of c-Myc and cyclin D1 in all cell lines examined (Fig. 2A), in agreement with previously published data (2, 12, 39, 42, 64). Changes in both cyclin D1 levels and the proportion of cells in S phase following E_2 treatment were smaller than we have reported previously (42), probably reflecting less marked cell cycle synchrony in these clonal cell lines. Zn induction of c-Myc in myc.3 cells had no effect on the expression of cyclin D1 from 3 to 24 h (Fig. 2A and data not shown). Similarly, Zn-induced expression of cyclin D1 in D1.13 cells had no effect on the expression of c-Myc from 3 to 24 h (Fig. 2A and data not shown). Furthermore, induced expression of c-Myc or cyclin D1 did not affect the expression of the other gene product in any other MCF-7.7 cell lines examined (Fig. 1A and data not shown).

The concentration-dependent induction of c-Myc and cyclin D1 by E_2 or Zn and the degree of S-phase entry were examined in representative cell lines. Treatment of myc.3 cells with 100 nM E_2 or 50 μM Zn resulted in similar levels of both S-phase entry (Fig. 1C and 2B) and c-Myc protein expression (Fig. 2). Similarly, treatment of D1.13 cells with E_2 or 50 μM Zn resulted in similar levels of S-phase entry (Fig. 1C and 2B). However, in marked contrast to the situation with c-Myc, a greater than twofold-higher level of cyclin D1 protein was required following Zn treatment to elicit the same degree of S-phase entry as that induced by E_2 treatment (Fig. 2). When Zn concentrations were adjusted to induce a level of cyclin D1 protein expression similar to that induced by 100 nM E_2, i.e., 30 μM Zn, the increase in S phase was only ~40% of that induced by E_2 (Fig. 2B). These data are consistent with a model of E_2 action in which E_2-induced expression of c-Myc, but not cyclin D1, is sufficient to account quantitatively for the subsequent S-phase entry. However, it is clear that the E_2-induced expression of cyclin D1 can still make a substantial contribution to S-phase entry.

Cdk4 is activated by induction of cyclin D1 but not c-Myc. Although increased expression of c-Myc was without effect on cyclin D1 expression, it is possible that the c-Myc pathway can activate cyclin D1-associated CDKs (56). The major contribution to cyclin D1-associated kinase activity in MCF-7 cells is from cyclin D1-Cdk4 complexes since in these cells cyclin D1-Cdk2 complexes are inactive and cyclin D1-Cdk6 complexes are in low abundance (59). E_2 treatment of all cell lines resulted in similar increases in the level of Cdk4 activity (Fig. 3A and data not shown). Zn treatment of D1.13 cells, but not

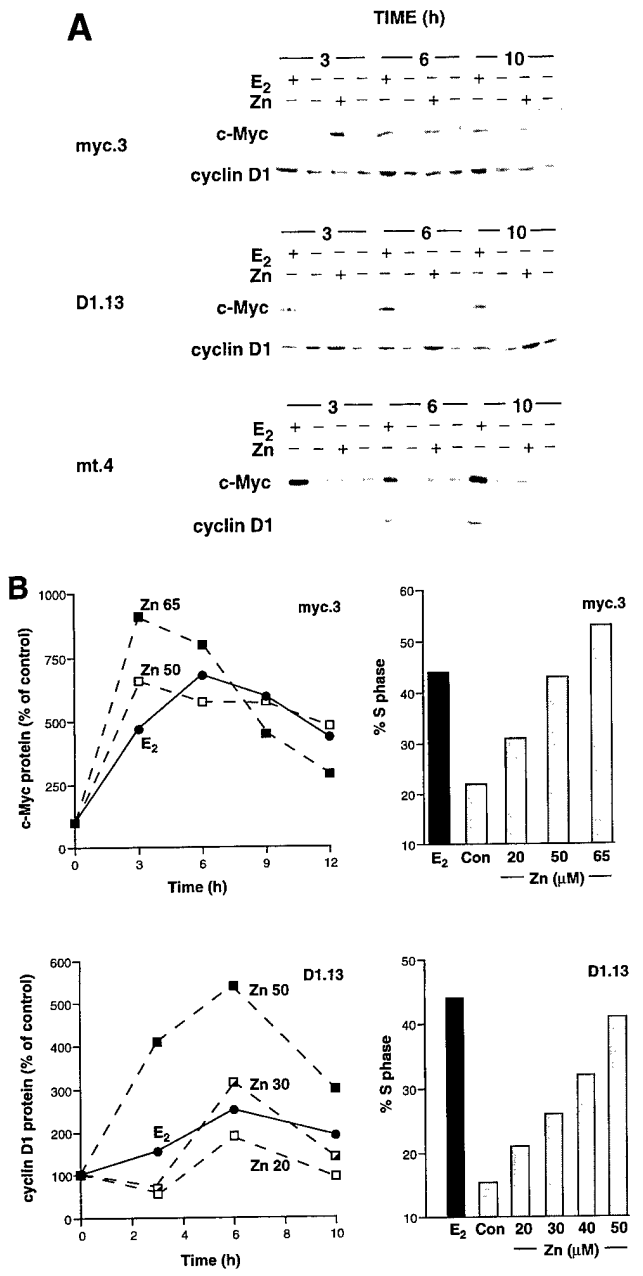

FIG. 2. c-Myc and cyclin D1 protein expression following E_2 treatment or Zn induction of c-Myc or cyclin D1. Three of the clonal MCF-7.7 cell lines used for Fig. 1 (myc.3, D1.13, and mt.4) were growth arrested with 10 nM ICI 182780 for 48 h. (A) Cells were treated at time zero with 50 μM Zn or 100 nM E_2 (+) or with vehicle (−). Whole-cell lysates were prepared at intervals thereafter (shown in hours). Lysates were immunoblotted with antibodies against c-Myc and cyclin D1. (B) Cells were treated at time zero with either the indicated concentration (micromolar) of Zn, 100 nM E_2, or vehicle (Con). At intervals thereafter, cell lysates were prepared and immunoblotted with antibodies against c-Myc or cyclin D1. Autoradiographs were quantitated by densitometry and expressed relative to time-matched controls. After 18 h (myc.3) or 21 h (D1.13), cells were harvested and stained for DNA content, and the proportion of cells in S phase was determined by flow cytometry. Data for protein and S phase are from the same experiment.

myc.3 cells, was accompanied by early activation of Cdk4 (Fig. 3A), paralleling the changes in cyclin D1 protein expression in these cell lines (Fig. 2A). Similarly, Cdk4-specific phosphorylation of pRB detected by immunoblot analysis with an antibody specific for a Ser-780 Cdk4 phosphorylation site on pRB (21) was substantially increased following Zn treatment of D1.13 cells (Fig. 3B). In contrast, in both myc.3 and mt.4 cells there were only small changes in Ser-780 pRB phosphorylation following Zn treatment (Fig. 3B), indicating that Zn treatment had minor effects on this parameter and c-Myc expression had no effect. E_2 treatment of all cell lines resulted in similar levels of Cdk4-specific phosphorylation of pRB at 16 h (Fig. 3B).

Induction of c-Myc or cyclin D1 leads to activation of cyclin E-Cdk2 and hyperphosphorylation of pocket proteins. The effect of c-Myc or cyclin D1 induction on cyclin E-Cdk2 activity was next examined since both have been reported to activate cyclin E-Cdk2 (34, 36, 48, 56). Activation of cyclin E-Cdk2 occurs relatively early after E_2 treatment (12, 39, 42), suggesting a particular importance for this kinase in E_2-induced G_1-S-phase progression. E_2 treatment of all cell lines and Zn induction of c-Myc or cyclin D1 were followed by activation of cyclin E-Cdk2, beginning with minor increases at 3 h (~20% [Fig. 4A]) and increasing thereafter. Cyclin E-Cdk2 activity reached levels ~4-fold above control levels at 16 h in cells treated with E_2 and ~3.5-fold above control levels at 16 h following Zn induction of c-Myc (Fig. 4A). In contrast, cyclin E-Cdk2 activity reached maximum levels at 6 h (~3-fold above control levels) following Zn induction of cyclin D1 and thereafter remained constant (Fig. 4A). Zn treatment of mt.4 control cells had little effect on cyclin E-Cdk2 activity. These results indicate that the E_2-activated c-Myc and cyclin D1 pathways converge at or prior to cyclin E-Cdk2 activation.

Since pocket proteins are in vivo substrates for G_1 CDKs, we next examined the phosphorylation of pRB, p130, and p107 by immunoblotting following E_2 treatment or Zn induction of c-Myc or cyclin D1. As expected from previous studies (42, 65),

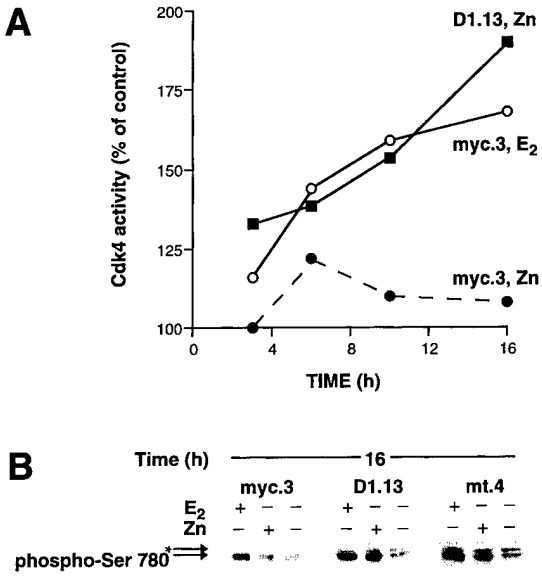

FIG. 3. Cdk4 activity following E_2 treatment or Zn induction of c-Myc or cyclin D1. The experimental design was as described for Fig. 2A. (A) Cdk4 immunoprecipitates were assayed for kinase activity toward a GST-pRB$^{773\text{-}928}$ substrate. Autoradiographs were quantitated by densitometry and expressed relative to time-matched controls. E_2 treatment of all cell lines resulted in similar levels of Cdk4 activity and is represented by results from myc.3 cells. Points shown represent the means of two independent experiments. (B) Total cell lysates were immunoblotted with antibodies against a pRB-derived phosphopeptide that contains a Cdk4-specific target (phospho-Ser 780). The immunoreactive band labeled with an asterisk is nonspecific since it was not detected in pRB immunoprecipitates (data not shown).

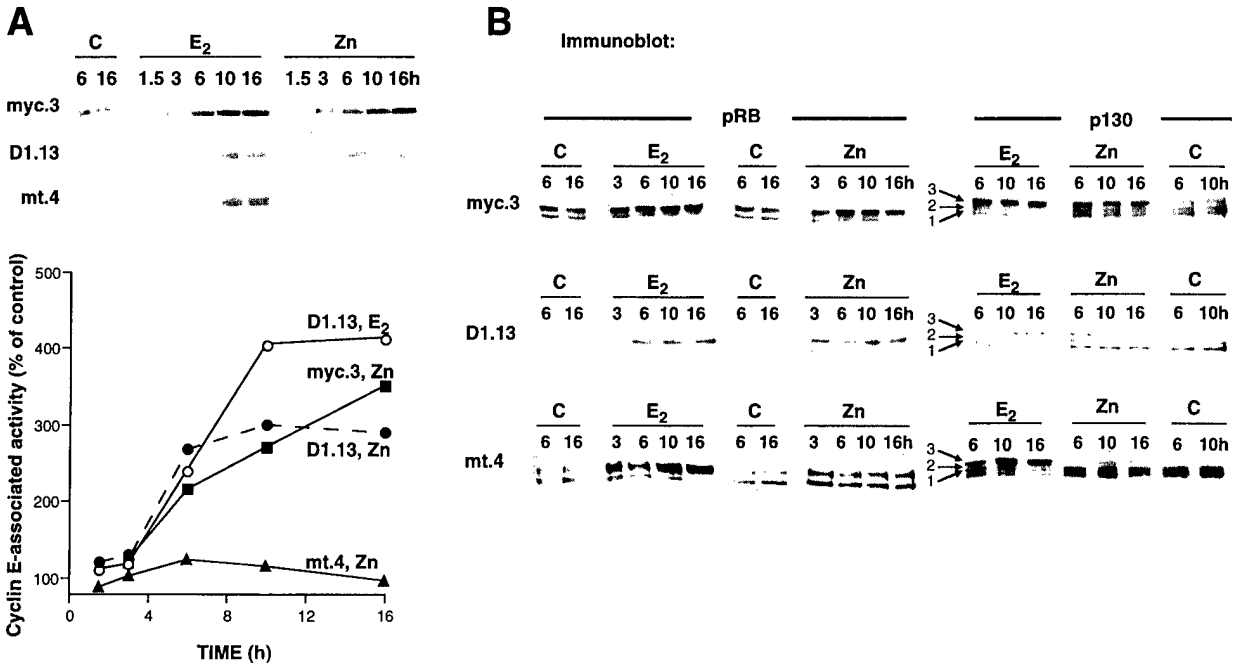

FIG. 4. Cyclin E-Cdk2 activation and hyperphosphorylation of pocket proteins following E_2 or Zn treatment. The experimental design was as described for Fig. 2A. Control lanes (C) represent results from cells treated with vehicle. (A) Cyclin E immunoprecipitates were assayed for kinase activity toward histone H1 substrate. A 1.5-h time point is included for D1.13 and mt.4 cells. For each cell line, the results shown are from the same autoradiograph. Autoradiographs were quantitated by densitometry, and results are expressed relative to those for time-matched controls. E_2 treatment of all cell lines resulted in similar levels of cyclin E-associated kinase activity and is represented by results for D1.13 cells. Points shown for 3 to 16 h represent the means of two independent experiments. (B) Cell lysates were immunoblotted with either pRB or p130 antibodies. Three distinct phosphorylated species of p130 are indicated. For each cell line and antibody the results shown are from the same autoradiograph.

a significant proportion of pRB was hypophosphorylated (most mobile form) following antiestrogen pretreatment (Fig. 4B). p130 was mainly present as hypophosphorylated form 1 and phosphorylated form 2 (27, 28), characteristic of G_0-phase cells. E_2 treatment of all cell lines resulted in an increase in the total amount of hyperphosphorylated, less mobile pRB and p130 (form 3) (Fig. 4B) and an increase in the hyperphosphorylated/hypophosphorylated ratio of pRB and p130. Zn induction of c-Myc in myc.3 cells resulted in similar phosphorylation of pRB but less pronounced phosphorylation of p130 (Fig. 4B). In contrast, Zn induction of cyclin D1 in D1.13 cells resulted in earlier phosphorylation of pRB and p130 (3 to 6 h) than E_2 treatment (6 to 10 h), consistent with the more rapid effects of Zn treatment on cyclin D1 protein expression (Fig. 2A), Cdk4 activity, and Cdk4-specific phosphorylation of pRB (data not shown). Similar to the effects of ectopic gene expression on pRB and p130, p107 phosphorylation was evident by 3 h in D1.13 cells, 6 h in myc.3 cells, and not at all in mt.4 cells following Zn treatment (data not shown). For all cell lines studied, E_2 treatment resulted in an increased degree of p107 phosphorylation that was evident by 6 h (data not shown).

Cells subjected to E_2- and c-Myc-induced, but not those subjected to cyclin D1-induced, G_1-S-phase progression have similar sensitivities to inhibition by roscovitine. The above experiments showed that similar degrees of G_1-S-phase progression following E_2 treatment or ectopic expression of c-Myc or cyclin D1 were preceded by induction of similar levels of cyclin E-Cdk2 activity. The dependence of G_1-S-phase progression on cyclin E-Cdk2 activity was next determined by examining sensitivity to the Cdk2-specific chemical inhibitor roscovitine (7, 29). The maximum increases in E_2- or Zn-induced S-phase entry were compared following treatment with different concentrations of roscovitine. Both myc.3 and D1.13 cell lines had similar sensitivities to roscovitine inhibition of E_2-induced G_1-S-phase progression, with ~40% inhibition of S-phase entry with 10 μM roscovitine and ~100% inhibition with 25 μM roscovitine (Fig. 5). G_1-S-phase progression following Zn induction of c-Myc in myc.3 cells was also inhibited with a similar sensitivity (Fig. 5). However, G_1-S-phase progression following Zn induction of cyclin D1 in D1.13 cells was markedly less sensitive to roscovitine, such that concentrations of roscovitine as high as 10 μM had no inhibitory effect (Fig. 5). These results demonstrate that G_1-S-phase progression stimulated by E_2 and c-Myc was similarly dependent on Cdk2 activity. However, the G_1-S-phase progression stimulated by cyclin D1 was markedly different, being less dependent on Cdk2 activity despite similar levels of cyclin E-Cdk2 activation. It is possible that the early increase in cyclin D1-Cdk4 activity following cyclin D1 induction (Fig. 3A) compensates for the loss of Cdk2 activity and thereby accounts for the relative resistance to roscovitine.

c-Myc- and cyclin D1-induced activation of cyclin E-Cdk2 is associated with loss of p21 and association with p130. The E_2-stimulated c-Myc and cyclin D1 pathways appeared to converge on or just prior to cyclin E-Cdk2 activation. Therefore, the mechanisms of cyclin E-Cdk2 activation were investigated to determine whether they were the same following c-Myc or cyclin D1 induction. In whole-cell lysates and cyclin E immunoprecipitates from myc.3 or D1.13 cells treated with E_2 or Zn, there were no changes in the levels of cyclin E, Cdk2, p21, or p27 from 0 to 16 h (data not shown), consistent with observations made following E_2 treatment of MCF-7 cells (39, 42). In these cells, cyclin E-Cdk2 activation is associated with the formation of high-specific-activity, high-molecular-weight cy-

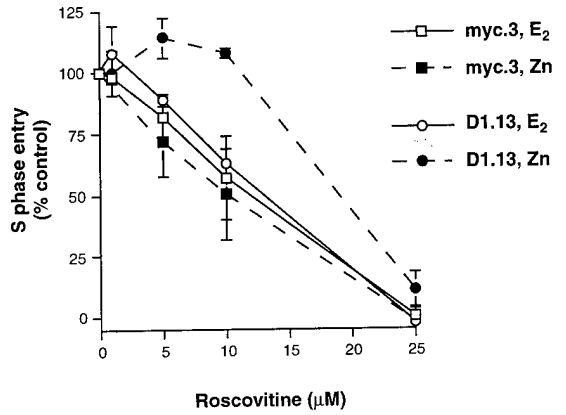

FIG. 5. Inhibition of E_2-, c-Myc-, or cyclin D1-induced G_1-S-phase progression by the Cdk2-specific inhibitor roscovitine. The experimental design was as described for Fig. 2A except that cells were pretreated with the indicated concentration of roscovitine 30 min prior to treatment with Zn, E_2, or vehicle. After 18 h (myc.3) or 21 h (D1.13), cells were harvested and stained for DNA content, and the proportion of cells in S phase was determined by flow cytometry. For each concentration of roscovitine the increase in S phase with either Zn or E_2 (above the vehicle-treated control level) was expressed as a percentage of the increase in S phase with 0 μM roscovitine. Points represent the means of three (myc.3) or four to five separate experiments (D1.13), and error bars indicate the standard errors of the means.

clin E-Cdk2 complexes lacking CDK inhibitors p21 and p27 (42). Gel filtration of cell lysates was therefore performed to determine if similar changes occurred following induction of c-Myc or cyclin D1. Zn treatment of myc.3 or D1.13 cells induced an increase in cyclin E-associated kinase activity that eluted between 400 and 500 kDa (fractions 1 and 2 [Fig. 6]). Subsequent experiments demonstrated less marked changes in cyclin E-associated kinase activity eluting at predicted molecular masses higher than 500 kDa (data not shown). These changes in the elution profile of cyclin E-associated kinase activity following c-Myc or cyclin D1 expression are similar to the changes following E_2 treatment of these clones (data not shown) and parental MCF-7 cells (42). The relatively greater proportion of high-molecular-weight cyclin E-associated kinase activity following expression of c-Myc compared to that following cyclin D1 expression is likely to indicate some differences in complex composition. In contrast, in all clones most of the cyclin E protein eluted at ~160 kDa (fraction 5) [Fig. 6 and 7B and data not shown]) as previously described (42). Consequently the specific activity of the 400- to 500-kDa cyclin E complexes was ~12-fold greater than that of the ~160-kDa complexes, demonstrating that the activity of the total cyclin E-Cdk2 pool was due to a small number of highly active high-molecular-weight complexes.

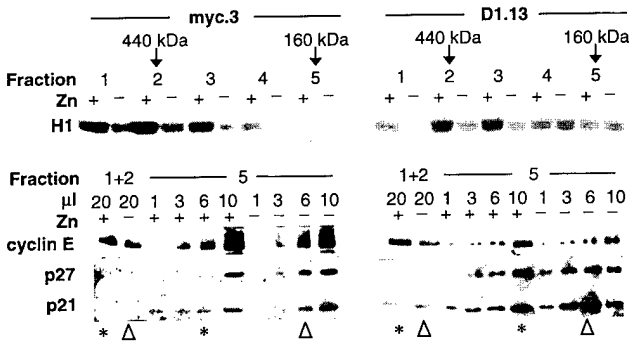

FIG. 6. Mechanism of activation of cyclin E-Cdk2 by E_2 treatment or Zn induction of c-Myc or cyclin D1. The experimental design was as described for Fig. 2A. Lysates from myc.3 and D1.13 cells were prepared 10 h after treatment with Zn and fractionated on a HiLoad 16/60 Superdex 200 gel filtration column. Cyclin E complexes were immunoprecipitated from 3-ml fractions and then either assayed for histone (H1) kinase activity (as described for Fig. 3A) or resuspended in 20 μl of sample buffer, separated by SDS-PAGE, transferred to a nitrocellulose filter, and sequentially blotted with the indicated antibodies. Fraction 5 (which contained high levels of cyclin E) was loaded in variable amounts in order to permit comparison of the relative levels of coimmunoprecipitating proteins with those in fractions 1 and 2 (combined). Lanes containing similar levels of cyclin E protein are indicated with either an asterisk (Zn treated) or an arrowhead (vehicle treated). The elution volumes for marker proteins of known molecular weight are indicated at the top.

The composition of the 400- to 500-kDa cyclin E complexes was compared with that of the relatively inactive ~160-kDa complexes. Cyclin E immunoprecipitates were prepared from fractions 1 and 2 (400 to 500 kDa) or fraction 5 (~160 kDa), resuspended in 20 μl of sample buffer, and then separated by SDS-PAGE. Since there was relatively little cyclin E protein eluting at 400 to 500 kDa compared to that eluting at ~160 kDa, different amounts of fraction 5 (~1, 3, 6, and 10 μl) were loaded on the gel to facilitate comparison of cyclin E/CDK inhibitor ratios. The relatively high level of cyclin E protein eluting at ~160 kDa is clearly evident (compare cyclin E protein in fractions 1 and 2 with all 4 lanes from fraction 5). In myc.3 and D1.13 cells, the 400- to 500-kDa cyclin E complexes were relatively deficient in p21 and p27 compared with lanes containing similar levels of cyclin E in the ~160-kDa complexes. These differences are likely to contribute to the high specific activity of the 400- to 500-kDa cyclin E complexes. Zn induction of c-Myc or cyclin D1 increased the levels of cyclin E eluting at 400 to 500 kDa without altering the levels of cyclin E-associated p21 or p27, indicating an increase in high-molecular-weight cyclin E complexes that were not associated with these CDK inhibitors. Consistent with this, there was an increase in the relative abundance of the more mobile, CAK (CDK-activating kinase)-phosphorylated form of cyclin E-associated Cdk2 species (13) in the 400- to 500-kDa complexes (data not shown), since CAK phosphorylation of Cdk2 is prevented by association of p21 and p27 with Cdk2 (3, 40). These changes in cyclin E complex composition were identical to the changes following E_2 treatment of these clones (data not shown) and MCF-7 cells (42).

The composition of the active cyclin E-Cdk2 complexes was investigated further by examining interactions between cyclin E and proteins previously found to associate with cyclin E. Significant interactions were detected between cyclin E and the pocket protein p130. In cyclin E immunoprecipitates, p130 was present predominantly in the hyperphosphorylated form 3, with little hypophosphorylated protein detectable (Fig. 7A). Following E_2 treatment, or Zn induction of c-Myc or cyclin D1, the levels of p130 associated with cyclin E increased. Consistent with these data, p130 immunoprecipitates contained increased levels of cyclin E and Cdk2 following all treatments (Fig. 7A). Taken together, these results indicated an increase in protein complexes containing cyclin E-Cdk2 and hyperphosphorylated p130. No significant interactions between cyclin E and the other pocket protein p107 or pRB were detected in similar experiments (data not shown). Cyclin E-Cdk2-p130 complexes eluted from the gel filtration column coincident with active cyclin E complexes (data not shown), suggesting that the active complexes may contain p130. Immunodepletion of p130 was sufficient to remove the majority of the cyclin E protein and cyclin E-Cdk2 activity that eluted at 400 to 500 kDa following E_2 and induction of c-Myc or cyclin D1 (Fig. 7B). This finding indicates that cyclin E-p130 complexes constitute the majority of 400- to 500-kDa cyclin E complexes, and

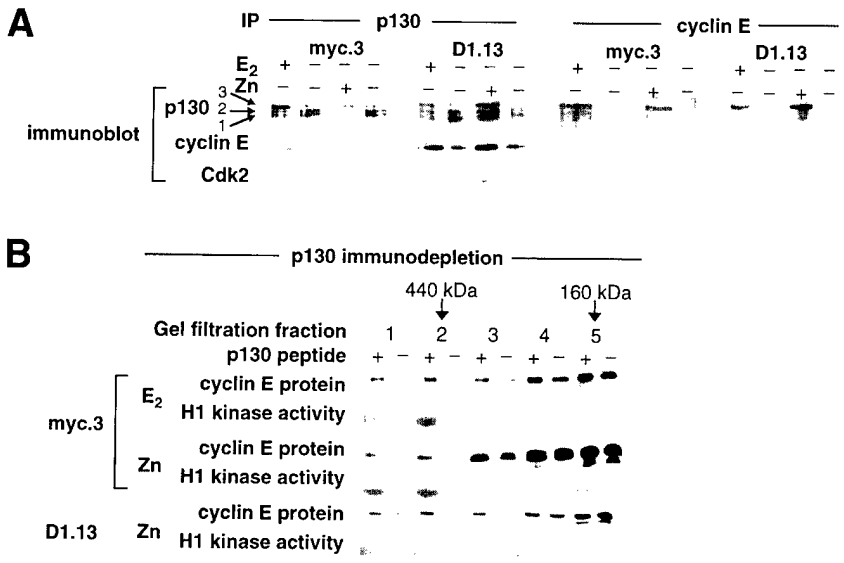

FIG. 7. Increased association of cyclin E with p130 follows E_2 treatment or Zn induction of c-Myc or cyclin D1. The experimental design was as described for Fig. 2A. Lysates were prepared 10 h after treatment. (A) Cyclin E or p130 immunoprecipitates (IP) from total cell lysates were immunoblotted with the indicated antibodies. (B) p130 was immunoprecipitated from lysates with p130 antibodies in the presence (+) or absence (−) of immunizing peptide. The supernatant was fractionated on a gel filtration column as described for Fig. 6. Representative cyclin E protein immunoblots and cyclin E histone (H1) kinase assays are shown following E_2 and Zn treatment of myc.3 cells and Zn treatment of D1.13 cells. The elution volumes for marker proteins of known molecular weight are indicated.

these complexes contribute most of the total cyclin E-Cdk2 activity. In summary, activation of cyclin E-Cdk2, whether by E_2, c-Myc, or cyclin D1, was invariably associated with the formation of high-molecular-weight cyclin E-Cdk2 complexes that were relatively deficient in both p21 and p27 and contained p130 and CAK-phosphorylated Cdk2.

E_2, c-Myc, and cyclin D1 decrease the association between p21 and recombinant cyclin E-Cdk2. The decrease in p21 association with cyclin E-Cdk2 complexes in vivo following E_2 treatment has been proposed as a major factor contributing to the relief of inhibition of cyclin E-Cdk2 (39, 42). The association between p21 and recombinant cyclin E-Cdk2 in vitro is also inhibited following E_2 treatment (42) and was therefore investigated in the current paradigm. Zn induction of c-Myc or cyclin D1 was accompanied by decreased association of p21 with cyclin E-Cdk2 complexes in vivo (Fig. 6) and reduced association between p21 and recombinant GST-cyclin E-Cdk2 in vitro (Fig. 8A). Control experiments showed no association between p21 and GST, indicating that the binding was specific for cyclin E-Cdk2 (data not shown). These results indicate that decreased association of p21 with cyclin E-Cdk2 is a common activating mechanism for cyclin E-Cdk2 shared by E_2, c-Myc, and cyclin D1. p21 levels do not change at the time of early activation of cyclin E-Cdk2 following E_2 treatment of MCF-7 cells (42). Similarly, levels of p21 did not alter following Zn induction of c-Myc or cyclin D1 (data not shown), and therefore decreased abundance of p21 does not appear to account for the decreased association of p21 with cyclin E-Cdk2. An alternative explanation for this effect is that p21 is sequestered by other proteins and thus is unavailable for binding to cyclin E-Cdk2. It has been suggested that cyclin D1-Cdk4 performs this role following E_2 treatment of MCF-7 cells (39). Examination of cyclin D1 immunoprecipitates revealed that there was increased association of cyclin D1 with p21 following E_2 treatment or Zn induction of cyclin D1 but not following Zn induction of c-Myc (Fig. 8B). This finding indicates that cyclin D1, but not c-Myc, may contribute to the activation of cyclin E-Cdk2 by sequestering p21 into cyclin D1 complexes.

DISCUSSION

E_2-induced G_1-phase progression can be mimicked by c-Myc or cyclin D1. The proliferative effect of estrogens is of major importance in the development and normal physiological function of female reproductive organs and in breast cancer initiation and progression. We and others have used the estrogen-responsive human breast cancer cell line MCF-7 to investigate the underlying molecular mechanisms for the proliferative effect. This study has focused on the roles of c-Myc and cyclin D1 in this process, since both gene products can stimulate cell cycle progression (17, 33, 44, 45) and the expression of both genes is rapidly induced following E_2 treatment (2, 10, 12, 39, 42). Our results demonstrate that ectopic expression of either c-Myc or cyclin D1 induced S-phase entry in MCF-7 cells previously arrested in G_1 phase by pretreatment with anties-

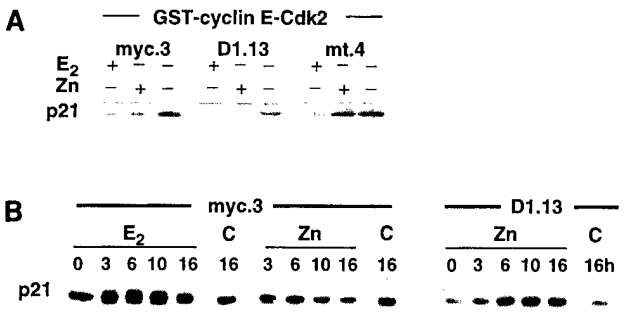

FIG. 8. Effects of E_2 treatment or Zn induction of c-Myc or cyclin D1 on the binding of p21 to recombinant cyclin E-Cdk2. The experimental design was as described for Fig. 2A. (A) Lysates prepared 10 h after treatment were incubated with GST-cyclin E-Cdk2 complexes or GST. GST proteins were recovered on glutathione-agarose beads and then immunoblotted for p21. (B) Cyclin D1 immunoprecipitates were immunoblotted for p21. Immunoblots of control nonimmune rabbit antiserum immunoprecipitates failed to detect p21. Control lanes (C) represent results from cells treated with vehicle. For each cell line the results shown are from the same radiograph.

trogen. c-Myc is therefore sufficient to initiate G_1-S-phase progression in this epithelial cell model, extending previous findings in other cell types. These data also indicate a potential role for c-Myc in clinical antiestrogen resistance, similar to the one we have suggested previously for cyclin D1 (67). In this model, c-Myc did not induce expression of cyclin D1 protein. Similarly, others have demonstrated that activation of conditional c-Myc alleles (MycER) does not activate cyclin D1 transcription (54), despite some conflicting earlier reports (5, 20). However, MycER activates cyclin D1-dependent CDKs in rat fibroblasts (43, 56), but the effect is relatively small in contrast to the large changes in both cyclin E-Cdk2 activity and G_1-S-phase progression. In our system, Zn induction of c-Myc did not increase Cdk4 activity, and conversely, Zn induction of cyclin D1 and subsequent Cdk4 activation did not induce expression of c-Myc. These results demonstrate that E_2-induced G_1-phase progression is likely to be mediated by initially distinct c-Myc and cyclin D1 pathways. It remains possible that the cyclin D1 pathway upregulates the activity of the c-Myc pathway at some point other than c-Myc protein expression.

Comparison of protein expression and S-phase entry following E_2 treatment or inducible expression of c-Myc or cyclin D1 suggested that the effects of E_2 in these cells were quantitatively more closely mimicked by induction of c-Myc than by induction of cyclin D1, indicating that E_2-induced expression of c-Myc may be sufficient for G_1-S-phase progression. Consistent with a predominant role for c-Myc over cyclin D1 in E_2-induced cell proliferation, S-phase entry induced by E_2 or c-Myc, but not by cyclin D1, was equally sensitive to inhibition by the Cdk2 antagonist roscovitine. c-Myc expression is apparently necessary for E_2-dependent G_1-S-phase progression in breast cancer cells, since c-Myc antisense oligonucleotides can prevent E_2-dependent MCF-7 cell proliferation (64). However, this observation does not preclude a requirement for cyclin D1 in E_2- and c-Myc-induced G_1-S-phase progression. In fibroblasts, cyclin D1 antibodies prevent c-Myc-induced S-phase entry (47), and the Cdk4/6 inhibitor p16 inhibits c-Myc-dependent transformation (14). Therefore, the functional consequences of c-Myc expression appear to be dependent on cyclin D1 expression. In MCF-7 cells, cyclin D1 protein levels are reduced by only 50% following antiestrogen pretreatment (42, 65) and may be sufficient for subsequent c-Myc action. Indeed, inhibition of cyclin D1 function in MCF-7 cells also inhibits E_2-dependent S-phase entry (26), although it remains to be determined whether the role of cyclin D1-Cdk4 complexes involves Cdk4 activity, CDK inhibitor sequestration, or some other function.

Cyclin E-Cdk2 is activated following c-Myc or cyclin D1 expression. Activation of cyclin E-Cdk2 is necessary for G_1-S-phase progression (35, 60, 62) and is inhibited in antiestrogen-arrested MCF-7 cells by association with p21 (39, 42). Expression of c-Myc or cyclin D1 resulted in early activation of cyclin E-Cdk2, and therefore the E_2-induced expression of c-Myc and cyclin D1 is likely to activate pathways that converge at or prior to this point. Activation of cyclin E-Cdk2 by E_2 or following c-Myc or cyclin D1 expression was associated with decreased p21 in high-molecular-weight, high-specific-activity cyclin E-Cdk2 complexes, suggesting a common mechanism of activation involving formation of cyclin E-Cdk2 complexes deficient in p21. Further evidence for such a mechanism is provided by increased CAK-phosphorylated Cdk2 in high-specific-activity cyclin E-Cdk2 complexes since CDK inhibitors directly prevent CAK phosphorylation of Cdk2 (3, 40). CAK activity (measured as Cdk7 activity) was unchanged following E_2 treatment of MCF-7 cells (42) and therefore is unlikely to account for the increase in CAK-phosphorylated Cdk2. Moreover, peptide motifs shared by p130 and p21 ensure that their binding to cyclin E-Cdk2 is mutually exclusive (1, 52), which suggests that active cyclin E-Cdk2 complexes which contain p130 are deficient in p21. Therefore, competition between proteins with these shared peptide motifs plays a major role in determining cyclin E-Cdk2 complex formation, activity, and substrate preference following E_2 treatment. To our knowledge, this is the first description of competition between p21 and p130 for association with cyclin E-Cdk2 in a physiologically relevant model.

There are a number of potential mechanisms for the formation of cyclin E-Cdk2 complexes that are deficient in p21 and contain p130. These include changes to any one of the proteins involved (cyclin E, Cdk2, p21, and p130) such that cyclin E-Cdk2-p130 complex formation is favored over cyclin E-Cdk2-p21 complex formation. Our in vitro binding studies demonstrate that binding of p21 to recombinant cyclin E-Cdk2 is decreased following E_2 treatment, c-Myc expression, or cyclin D1 expression. Therefore, it is likely that changes in p21 rather than changes in cyclin E-Cdk2 account for the alteration in complex formation. Furthermore, changes to p130 are unlikely to account for the alteration because binding of p21 to recombinant cyclin E-Cdk2 is not altered by p130 immunodepletion (41). These data argue that E_2 treatment, c-Myc expression, or cyclin D1 expression may instead target p21 and prevent its association with cyclin E-Cdk2, for example, by phosphorylation/sequestration of p21 or decreased production or increased destruction of the pool of p21 capable of binding to cyclin E-Cdk2. Increased binding of p21 to cyclin D1-Cdk4 occurred following E_2 treatment and cyclin D1 induction, and cyclin D1-Cdk4 may therefore sequester p21 from cyclin E-Cdk2. Our observation that c-Myc can promote G_1-S-phase progression in the absence of an increase in Cdk4 activity may indicate that the major role for cyclin D1 in E_2 action is sequestration of p21 rather than activation of Cdk4, as has been suggested by others (39). However, binding of p21 to cyclin D1-Cdk4 did not increase following c-Myc induction indicating a different activating mechanism by c-Myc. These observations are similar to those made for rat fibroblasts, in which c-Myc activated cyclin E-Cdk2 by inhibiting association with p27 and without sequestration of p27 by cyclin D1 (36, 63). c-Myc has also been reported to abrogate the inhibitory action of p21 on Cdk2 activity in fibroblasts (18). Potentially, c-Myc may target all members of the p21/p27 class of CDK inhibitors and prevent their association with cyclin E-Cdk2 by a common mechanism.

Cyclin E-Cdk2 complexes activated by E_2 treatment, or expression of c-Myc or cyclin D1, are associated with p130. The c-Myc and cyclin D1 pathways also converged on p130 phosphorylation. Following antiestrogen pretreatment, p130 was present as the faster-migrating phosphorylated forms 1 and 2 and formed complexes that contained E2F-4 but lacked cyclin E-Cdk2 (41). Similar complexes and phosphorylated forms of p130 are typical of G_0-phase cells derived from populations of normal and immortalized cells and from cancer cell lines (4, 27, 28, 30, 61). Following E_2 treatment, p130 was phosphorylated to the more slowly migrating form 3. This pattern is similar to that following cell cycle reentry stimulated by serum (27). These observations are consistent with earlier observations on E_2 action (reviewed in reference 58) which demonstrate both recruitment of noncycling cells into the cell cycle and acceleration of G_1-phase progression. Phosphorylation of p130 is likely to be due to cyclin E-Cdk2 since p130 phosphorylation coincided with both activation of cyclin E-Cdk2 and formation of cyclin E-Cdk2-p130 complexes. Cdk2 can phosphorylate p130 in vitro (28, 68), and both cyclin E-Cdk2 and

cyclin A-Cdk2 are capable of phosphorylating associated p130 (15, 24, 69). The p130-containing complexes contributed substantially to cyclin E-Cdk2 histone kinase activity since p130 immunodepletion of lysates prior to gel filtration resulted in a significant diminution of cyclin E-associated kinase activity. Others have reported increases in p130-associated histone kinase activity during G_1-phase progression (27, 68). However, p130 has also recently been reported to inhibit cyclin E-Cdk2 histone kinase activity (9, 69) and to redirect cyclin E-Cdk2 substrate specificity from histone to pocket protein family members (15), although the degree of phosphorylation of p130 in these studies was undefined. It is possible that phosphorylated p130 is less potent than hypophosphorylated p130 at inhibiting cyclin E-Cdk2 histone kinase activity, and this could account for the histone kinase activity associated with cyclin E-Cdk2-p130 complexes following E_2 treatment and following c-Myc or cyclin D1 expression.

Finally, these results support the presence of an undefined G_1-phase rate-limiting step in these cells, as the timing of S-phase entry was not closely tied to CDK activation or pocket protein phosphorylation. Cdk4 activation had increased by 3 to 6 h following cyclin D1 induction and did not increase at all following c-Myc induction. Pocket protein phosphorylation was almost complete by 3 to 6 h following cyclin D1 induction and by 10 to 16 h following either E_2 treatment or c-Myc induction, but the timing of S-phase entry was approximately the same following all treatments. Conversely, cyclin E-Cdk2 activation occurred by 3 to 6 h with all treatments, but cells did not enter S phase until 9 to 12 h after cyclin E-Cdk2 activation. Taken together, these data demonstrate that S-phase entry was still delayed despite the completion of a number of known rate-limiting steps including c-Myc expression, G_1-phase CDK activation, and pocket protein phosphorylation. A recent report demonstrates that cell size in fibroblasts is a requirement for S-phase entry despite c-Myc-induced activation of CDKs and hyperphosphorylation of pRB (43). A similar requirement for a critical cell size may represent the undefined G_1-phase rate-limiting step identified here, and this requires further investigation.

In conclusion, we have identified that in antiestrogen-arrested MCF-7 cells, increased expression of c-Myc and that of cyclin D1 are separate events activating pathways that are initially distinct. It is likely that both of these pathways contribute to E_2-induced G_1-S-phase progression, and our results support a predominant role for c-Myc in mediating estrogenic actions. The convergence of these pathways on the formation of active cyclin E-Cdk2 complexes deficient in p21 highlights a fundamental role for p21 in E_2 action.

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Estrogen Regulation of Cell Cycle Progression in Breast Cancer Cells

Owen W. J. Prall, Eileen M. Rogan and Robert L. Sutherland*

Cancer Research Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Darlinghurst, Sydney, NSW 2010, Australia

Estrogens are potent mitogens in a number of target tissues including the mammary gland where they play a pivotal role in the development and progression of mammary carcinoma. The demonstration that estrogen-induced mitogenesis is associated with the recruitment of non-cycling, G_0, cells into the cell cycle and an increased rate of progression through G_1 phase, has focused attention on the estrogenic regulation of molecules with a known role in the control of G_1–S phase progression. These experiments provide compelling evidence that estrogens regulate the expression and function of c-Myc and cyclin D1 and activate cyclin E-Cdk2 complexes, all of which are rate limiting for progression from G_1 to S phase. Furthermore, these studies reveal a novel mechanism of activation of cyclin E-Cdk2 complexes whereby estrogens promote the formation of high molecular weight complexes lacking the CDK inhibitor p21. Inducible expression of either c-Myc or cyclin D1 can mimic the effects of estrogen in activating the cyclin E-Cdk2 complexes and promoting S phase entry, providing evidence for distinct c-Myc and cyclin D1 pathways in estrogen-induced mitogenesis which converge on the activation of cyclin E-Cdk2. These data provide new mechanistic insights into the known mitogenic effects of estrogens and identify potential downstream targets that contribute to their role in oncogenesis. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Sex steroid hormones have a major role in the growth and development of target tissues including the mammary gland where they interact with other hormones, growth factors and cytokines in the precise regulation of proliferation and differentiation. Although the molecular basis of these interactions remains poorly understood increasing insight into the steroidal regulation of proliferation has been gained using steroid responsive human breast cancer cell lines as models. In these cells estrogens are mitogenic [1] as a result of accelerated progression through G_1 phase of the cell cycle [2]. Similar mechanisms have been identified *in vivo* where an additional effect of estrogen in recruiting non-cycling, G_0, cells into the cell cycle has been documented [3]. These cell cycle phase-specific effects of estrogens have focused attention on the role of estrogens and their receptors in the control of key regulatory processes controlling the entry into, progression through, and exit from G_1 phase of the cell cycle.

Progress through G_1 phase requires phosphorylation and inactivation of the retinoblastoma protein (pRB) with the consequent release of E2F transcription factors essential for the activation of genes required for S phase progression [4, 5]. Phosphorylation of pRB is mediated by the action of holoenzyme complexes comprising a cyclin regulatory subunit and a catalytic cyclin-dependent kinase (CDK) [6, 7]. Control of CDK activity in G_1 phase and subsequent progression to S phase is achieved by several mechanisms including: transcriptional activation of D-type cyclins and cyclin E; activation and inactivation of cyclin/CDK enzyme complexes by phosphorylation/dephosphorylation events, mediated predominantly by the CDK activating kinase (CAK) and Cdc25 phosphatases [7]; and by interactions with members of two distinct families of CDK inhibitors

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*Correspondence to R. L. Sutherland. Tel. 9295 8322; Fax: 9295 8321; e-mail r.sutherland@garvan.unsw.edu.au.

of which $p16^{INK4A}$ and $p21^{WAF1,CIP1,SDI1}$ are prototypic [8].

Another well studied target of estrogen action with a role in the control of cell cycle progression is the proto-oncogene c-*myc*. c-*myc* is a central regulator of cell proliferation and apoptosis and encodes a nuclear phosphoprotein (c-Myc) of the basic helix-loop-helix family of transcription factors which is required for mitogenic signaling by growth factor receptors. In fibroblasts inhibition of c-Myc can cause G_1 cell cycle arrest (reviewed in [9]), whereas the activation of conditional alleles of c-*myc* in these cells results in activation of cyclin D1-Cdk4 and cyclin E-Cdk2 [10–12]. c-Myc is rapidly upregulated by estrogen in breast cancer cells, suggesting a similar importance in estrogen-regulated cell cycle progression.

The recent expansion of knowledge on the molecular mechanisms regulating rates of cell cycle progression has provided a framework within which to develop deeper insight into the mechanistic basis of estrogen-induced mitogenesis. This brief review summarizes recent developments in this area.

EXPERIMENTAL MODELS

Several studies investigating the effects of estrogens on cell proliferation have addressed effects on cell cycle entry and progression. The earliest studies involved the rodent uterus and mammary gland *in vivo* where estrogen increased the proportion of cells synthesizing DNA by recruiting noncycling cells into the cell cycle and reducing the duration of G_1 phase in cells that were already cycling (reviewed in [3]). More detailed information on cell cycle regulation by sex steroids has emanated from cell culture studies using steroid-responsive breast cancer cells. The growth inhibitory effects of antiestrogens in estrogen receptor (ER)-positive breast cancer cells are profound and demonstrate a G_1 phase action for these compounds [13, 14]. More precise mapping of the point of action of antiestrogens using cells synchronized by mitotic selection confined this to early-to-mid G_1 phase [15].

Estrogens would be expected to exert opposite effects on the same targets given that antiestrogens are competitive inhibitors of the ER-mediated actions of estrogens [16]. However, the growth stimulatory effects of estrogens in cell culture have often been subtle and heavily dependent on the experimental system employed. This appears to be due, in part, to the difficulties in inducing increased growth rates in cells that are already proliferating at near maximal rates in serum-supplemented medium [13, 17], and the requirement for other growth factors, particularly insulin/IGF-1, under serum-free or growth factor-depleted conditions [18]. Thus in an attempt to maximize stimulation of cell cycle progression by estrogen, several methods of cell synchronization have been employed to increase the proportion of cells in the estrogen-sensitive G_1 phase of the cell cycle [2, 19–22]. Leung *et al.* [2] used cells synchronized at either G_1/S (by double thymidine block) or G_2/M (by treatment with nocodazole) phase boundaries to assess the effects of estrogen on progression through different phases of the cell cycle and concluded that cells were most sensitive in early G_1 phase, immediately following mitosis. Similar conclusions can be drawn from studies in which MCF-7 cells were growth-arrested in G_1 phase by methods involving hydoxymethylglutaryl-CoA reductase inhibitors [20] or amino acid deprivation [19, 23], although the potentially confounding effects of these pretreatments needs to be considered. Several very recent studies [21, 22, 24] have demonstrated that breast cancer cells, growth-arrested in G_1 phase by pretreatment with antiestrogens, can then be stimulated to progress semi-synchronously through the remainder of G_1 phase and enter S phase following restimulation with estrogen. The use of specific estrogen antagonists to achieve cell synchrony in these experiments permits selective magnification of ER-mediated effects compared with more generalized responses to cell cycle progression. In summary, the data from these *in vitro* and *in vivo* experimental systems are compatible with a model whereby estrogens and antiestrogens acting via the ER regulate the rate of progression through early G_1 phase of the cell cycle.

REGULATION OF c-MYC EXPRESSION

Regulation of the proto-oncogene c-*myc* is amongst the earliest detectable responses to estrogens and antiestrogens, being apparent within 30 min [25]. The mitogenic, apoptotic and oncogenic functions of c-Myc depend upon dimerization with the heterologous protein Max, DNA-binding and transactivation, suggesting that c-Myc transforms cells by activating genes involved in cell proliferation and/or apoptosis (reviewed in [26]).

c-*myc* is apparently directly transcriptionally regulated by estrogen via an atypical estrogen response element [27] in contrast to the response to growth factor mitogens where c-Myc activation is the end result of a cascade of signaling events. In rat uteri [28] and breast cancer cell lines [25, 29] c-*myc* is regulated by estrogen with kinetics which mimic those following treatment of growth-arrested cells with peptide mitogens in other cell types. Furthermore, c-Myc antisense oligonucleotides inhibit estrogen-stimulated breast cancer cell proliferation [30] providing strong evidence that c-Myc is likely to play a key role in estrogen action. Studies using a steroid-inducible promoter (MMTV) have demonstrated that overexpression of c-Myc in mammary epithelium *in vivo* causes generalized hyperplasia [31, 32], apparently mimicking the response to overexpression *in*

vitro. Similarly, constitutive expression of v-*myc* in transgenic mammary tissue caused generalized hyperplasia of the glandular epithelium [33].

Recent studies implicate cyclin/CDK complexes as downstream targets of the mitogenic effects of c-Myc. In fibroblasts, c-Myc is both necessary and sufficient for G_1–S phase progression [9] and in these cells activation of conditional alleles of c-*myc* is followed by the activation of both cyclin D1-Cdk4 and cyclin E-Cdk2 and induction of DNA synthesis [10–12, 34]. c-Myc-activation of cyclin E-Cdk2 complexes can occur without large alterations in levels of the component proteins, prompting the search for alternative mechanisms as discussed later.

REGULATION OF CYCLIN D1 EXPRESSION AND FUNCTION

Treatment of breast cancer cells with antiestrogens inhibits pRB phosphorylation [35] while estrogen induces significant increases in the phosphorylation of this key substrate of cyclin-CDK complexes [19–22, 36]. This indicates that the cyclin-CDK complexes active in G_1 phase are likely to be targets of estrogen action. In particular, cyclin D1, which binds to and activates both Cdk4 and Cdk6, has been implicated in estrogen-induced cell cycle progression. D-type cyclins are induced as delayed-early response genes by a variety of mitogens in many cell types, and removal of growth factors in G_1 phase leads to rapid downregulation of D-type cyclins [37], consistent with the notion that these cyclins act as mitogenic sensors linking extracellular signals with cell cycle progression [6]. An essential role for cyclin D1 in mammary gland development is demonstrated by the absence of lobular–alveolar structures in mice with disruption of the cyclin D1 gene [38, 39]. In breast cancer cells D-type cyclins appear to play a role in mediating the effects of a diverse group of mitogens including growth factors and steroid hormones [40], and the abundance of cyclin D1 declines rapidly following exposure to growth inhibitory antiestrogens [35, 40].

Estradiol treatment of MCF-7 breast cancer cells that have been growth arrested by a variety of strategies is followed by pronounced increases in cyclin D1 protein expression beginning within 3 h, attaining maximal levels after 6–10 h and thereafter decreasing [19–22]. The increase in cyclin D1 expression occurs after the earliest changes in c-Myc expression, and is accompanied by increases in cyclin D1-Cdk4 association and Cdk4 activity [19–21]. Cyclin D1 upregulation also coincides with increased phosphorylation of pRB and precedes S phase entry by some 9 h, consistent with the possibility that these changes may be consequences of increased cyclin D1 expression.

The effect of estrogen on cyclin D1 protein expression appears to be predominantly transcriptionally mediated, since increased expression of cyclin D1 mRNA precedes changes in cyclin D1 protein [20, 21]. Inhibitors of protein synthesis block the increase in cyclin D1 mRNA [20, 21] and an estrogen responsive region within the first 944 bp upstream of the cyclin D1 startsite does not contain classical ERE sequences [20]. These data indicate that in contrast to the direct transcriptional upregulation by estrogen-ER of c-*myc* mRNA, the increased expression of cyclin D1 mRNA is indirect, although the *trans*-acting elements remain to be defined.

Compelling evidence that cyclin D1 plays an essential role in estrogen-induced cell cycle progression comes from studies in which cyclin D1 is either functionally inhibited, or its expression enforced ectopically. Thus when either antibodies against cyclin D1, or the Cdk4-specific inhibitor $p16^{INK4A}$ are introduced into MCF-7 cells by microinjection, estrogen fails to stimulate G_1–S phase progression [41], indicating that cyclin D1 is necessary for estrogen action. Conversely, induced expression of cyclin D1 in MCF-7 or T-47D breast cancer cells that have been growth-arrested in G_1 phase by pretreatment with antiestrogens is followed by Cdk4 activation, increased phosphorylation of pRB and subsequent S phase entry, thereby mimicking the actions of estrogen [24,46]. However, in this model, induction of cyclin D1 to a quantitatively similar level as that following estrogen treatment results in only about 50% of the S phase entry induced by estrogen [46]. Therefore the estrogen-induced expression of cyclin D1 may not be sufficient to account for all the proliferative effects of estrogen, indicating the presence of other important targets of estrogen, including c-Myc as discussed above.

ESTROGEN ACTIVATION OF CYCLIN E-CDK2 HOLOENZYME COMPLEXES

In addition to activation of cyclin D1-Cdk4 complexes, estrogen also activates cyclin E-Cdk2 complexes at times compatible with a role in pRB phosphorylation. Cyclin E, like cyclin D1, is also a major regulator of cell cycle progression, and is both necessary and rate-limiting for the G_1–S phase transition [42–44]. Activation of cyclin E-Cdk2 following estrogen treatment begins within the first 3 h, and continues to increase until S phase entry which begins after 12 h [19, 21, 22]. This is in contrast to cell cycle progression stimulated by other mitogens in which cyclin E-Cdk2 activation is associated with G_1–S phase transition [45] and suggests that cyclin E-Cdk2 has a particularly important role in estrogen-induced cell cycle progression. However, following estrogen treatment there is little or no change in the levels of cyclin E, Cdk2, or the CDK inhibitors, p21

and p27 in either total cell lysates or the cyclin E-Cdk2 complexes prior to entry into S phase [21]. Therefore it is perhaps surprising that cyclin E-Cdk2 activity is elevated within the first few hours of the estrogenic response, and the mechanistic basis for this effect has been explored in detail in two recent publications [21, 22].

Separation of the cyclin E-Cdk2 complexes by gel filtration chromatography indicated that estrogen treatment was associated with the formation of high molecular weight complexes [21]. These complexes constituted a minority of the cyclin E-Cdk2 protein but were of high specific activity, accounting for the majority of cyclin E-Cdk2 activity. This increased activity, which was associated with a shift in Cdk2 to the enzymatically active, threonine 160-phosphorylated form, could be accounted for by the relative deficiency of p21 and p27 in the high molecular weight complex. These data are consistent with a mechanism of activation of cyclin E-Cdk2 involving both reduced CDK inhibitor association and CAK-mediated phosphorylation of Cdk2. These data implicate redistribution of p21 and p27 rather than regulation of the levels of these proteins as a critical event in the early proliferative response to estrogen. A predominant role for p21 in this effect is suggested by the demonstration that estrogen treatment relieves an inhibitory activity toward cyclin E-Cdk2, and that this inhibitory activity is mediated by p21, not p27 [21, 22].

Further mechanistic insights into the activation of cyclin E-Cdk2 by estrogen have been provided by studies utilizing MCF-7 cells that contain either inducible c-Myc or cyclin D1. Similar to the situation following estrogen treatment, expression of c-Myc or cyclin D1 is sufficient to activate cyclin E-Cdk2 by promoting the formation of high molecular weight complexes lacking the CDK inhibitor p21 [46]. c-Myc expression was not accompanied by increased cyclin D1 expression or Cdk4 activation, nor was cyclin D1 induction accompanied by increases in c-Myc [46]. Similarly, others have demonstrated that activation of conditional c-Myc alleles (MycER) does not activate cyclin D1 transcription [47] despite some conflicting earlier reports [48, 49]. Together these results suggest that estrogen upregulates separate c-Myc and cyclin D1 pathways, which then converge on the activation of cyclin E-Cdk2.

A remaining major unanswered question is the mechanistic basis for the distribution of inhibitors between the active and inactive complexes. One potential explanation is that estrogen-induced accumulation of cyclin D1-Cdk4 complexes sequesters p21 thereby reducing its association with cyclin E-Cdk2 [22]. Alternatively estrogen may alter the properties of p21, reducing its ability to bind to the complex, perhaps as a result of increased c-Myc expression. A similar mechanism has been proposed to account for c-Myc-mediated activation of cyclin E-Cdk2 in fibroblasts, involving prevention of the association between the CDK inhibitor $p27^{KIP1}$ and cyclin E-Cdk2 [12, 34]. Recent evidence that the pRB-related proteins, p107 and p130, can compete with p21 for cyclin-CDK binding [50, 51] raises the possibility that these proteins may be recruited to the cyclin E-Cdk2 complex following estrogen treatment, contributing to the increased size of the complex and the decreased association with p21 [46].

CONCLUSIONS

Estrogens exert potent mitogenic effects on ER-positive mammary epithelial cells which are mediated predominantly in the G_1 phase of the cell cycle. The recent development of a powerful *in vitro* model system, wherein breast cancer cells are growth-arrested with a pure antiestrogen and cell cycle progression reinitiated with estrogen, has facilitated dissection of some early molecular events in estrogen action [21,

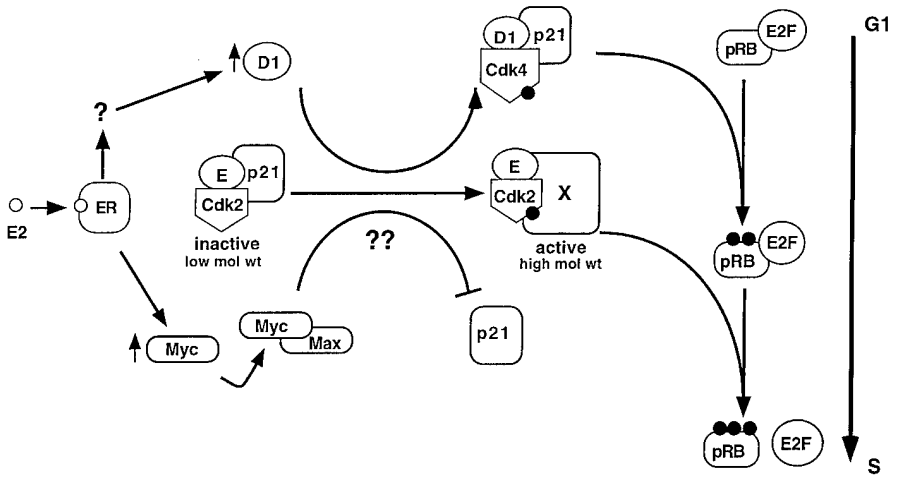

Fig. 1. A model of estrogen effects on molecules regulating G_1 phase progression. E2 (○), estrogen; ER, estrogen receptor; D1, cyclin D1: E, cyclin E; X, unknown protein(s) (●).

22]. A schematic representation of current knowledge developed from this model and the results of others is presented in Fig. 1. This indicates that the mitogenic effects of estrogen appear to be mediated by at least two, apparently distinct pathways, of which c-Myc and cyclin D1 respectively, are the key regulators. Transcriptional activation of c-*myc* is rapid and thought to be directly mediated via interaction with the ER. In contrast, estrogen stimulation of cyclin D1 expression requires the *de novo* synthesis of, as yet unidentified, intermediate proteins. Increased expression of cyclin D1 leads to formation of active complexes with Cdk4 and phosphorylation of pRB.

The net result of estrogen-induced c-Myc or cyclin D1 expression is early activation of the cyclin E-Cdk2 holoenzyme by the formation of high molecular weight cyclin E-Cdk2 complexes deficient in the CDK inhibitor p21 [21, 22]. This process appears to involve redistribution of p21 away from a small proportion of the total cellular cyclin E-Cdk2 complexes. Two potential mechanisms have been invoked to account for this redistribution involving, respectively, sequestration by cyclin D1-Cdk4 [22], or by an as yet undefined c-Myc-induced process. Subsequently, the cyclin E-Cdk2 complexes acquire a higher molecular weight (presumably due to association with an unidentified protein(s) labelled "X" Fig. 1) and high specific catalytic activity. Phosphorylation of pRB is a primary target of cyclin E-Cdk2 activity, resulting in the well-documented release of E2F transcription factors necessary for DNA synthesis, and progression from G_1 to S phase of the cell cycle.

Further studies are required to validate aspects of this model with an aim to providing a detailed mechanistic basis for the known mitogenic effects of estrogen in several target tissues.

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